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1. Studies were initiated to help estimate the probability of release of microorganisms from solids when fractured by impact. Spores of Bacillus subtilis var. niger were incorporated at various concentrations into lucite discs 15 mm in diameter and 2 mm thick. After surface sterilization each disc was aseptically fractured along a single plane and placed in a tube of trypticase soy broth (TSB), incubated at 32 C and observed for growth for 1 month. The number of discs that produced growth expressed as a fraction of the total tested was considered to indicate the probability of release of at least one viable spore from the lucite. The range of concentration in which the probability of release is greater than 0 and less than 1.0 has been located. Data will be collected in this range to determine the slope of the line representing probability of release as a function of spore concentration. In subsequent studies similar data will be obtained after test discs are fractured along more than one plane.

2. Studies on the enumeration of microbial contaminants on surfaces were continued. It was reported previously that recovery of B. subtilis var. niger spores from stainless steel strips by ultrasonics is improved when cold (4 C), rather than warm (25 C), peptone water is used. Similar experiments with naturally-occurring airborne microorganisms showed the reverse situation. Studies were made on the recovery of naturally-occurring contaminants from surfaces exposed to both cold (4 C) and warm (25 C) peptone water. In some instances the quantity of rinse water was varied to see if this might influence the recovery rates. A higher mean number of viable microorganisms was recovered at 25 C than at 4 C, and some of the differences were statistically significant. The recovery of fewer viable microorganisms when cold peptone water was used appeared to be attributable to a physical factor, rather than a biological one. Dust and particulate matter which settled onto stainless steel surfaces may have contained oily films which might be dispersed more easily in a warm solution than a cold one. Future studies will include the addition of Tween 80 and similar solutions to cold peptone water to determine if this will aid in reducing surface tension and thereby increase the efficiency of the recovery method.

   Studies of the acceptability of ultrasonic baths for use with the "Standard Procedures for the Microbiological Examination of Space Hardware" were continued. After testing several devices, a satisfactory method for physically measuring the ability of a unit to remove microbiological contamination from surfaces is yet to be found. Efforts are being made to develop a test using microbiological indicators; however, results to date have been too inconsistent to be acceptable as a standard.

   Correspondence was established with Mr. Frank LeDoux at the Goddard Space Flight Center to see if toroidal transformers or other components could be seeded artificially with B. subtilis var. niger spores. If components could be inoculated with known numbers of spores, a more reliable evaluation could be made of the recovery techniques used to determine the number of viable microorganisms present in similar components.

3. Studies on the recovery of sublethally-injured microorganisms were continued. Spores of B. subtilis var. niger, B. subtilis 5230 and Clostridium roseum were exposed to 600 mg/L of ethylene oxide for a period long enough to kill most but not all of them, and surviving organisms were recovered by standard procedures. Rinse fluids were plated with trypticase soy agar (TSA) and inoculated into tubes of TSB. It was shown that extended incubation at 32 C produced no significant increase in colony counts or in the number of positive tubes over that obtained after 3 days. Similar experiments were performed using as test organisms naturally-occurring spores which were separated from soil as described earlier (Quarterly Report No. 17). The results were similar to those obtained with pure spore suspensions. No significant increase in viable counts or positive tubes occurred after 3 days of incubation (Tables 1 and 2). In some cases a few broth cultures, which were negative after 7 to 14 days, were positive after 28 days of incubation. This phenomenon would be important if a sterility test were involved, but for purposes of enumeration by plate counts or multiple tube dilutions no significant difference in the estimation of bacterial spores would result from extended incubation beyond 3 days.

4. Studies were continued to determine the relative efficiency of several culture media in recovering anaerobic spores injured by dry heat. Spores of C. roseum and a species of Clostridium (SC-1), originally isolated from a spacecraft assembly area, were used as the test organisms. Stainless steel strips were inoculated with a spore suspension and dried for 16 hours under vacuum in the presence of silica gel. The seeded strips were then placed in a dry heat oven at 125 C long enough to kill most but not all the spores. After heating, the strips were placed in chilled sterile buffered distilled water and insonated for 12 minutes in an ultrasonic bath. The rinse fluids were pooled and appropriate dilutions made so that the number of viable spores, as determined with TSA was approximately 100 to 1,000 per ml. Control strips were processed in the same manner except that they were not exposed to dry heat. The test media are listed in Table 3. Several tests were performed to compare the efficiency of solid and liquid media for recovering anaerobic spores. Plate counts were done in quintuplicate and five series of multiple tube dilutions were used. A series consisted of five tubes for each of three decimal dilutions of the spore suspension. Most probable numbers (MPN) were computed (Standard Methods for the Examination of Water and Wastewater, including Bottom Sediments and Sludges, American Public Health Association, New York, N. Y. 12th Ed. 1965) based on presence or absence of visible growth after 72 hours of incubation at 32 C.

Table 4 shows that the pork infusion thioglycolate agar (PIT) and pork and pea agar (PPA) were about the same for recovering anaerobic spores of Clostridium sp. SC-1. When two batches of PPA, made up separately, were compared there appeared to be some variation in the levels of spores recovered, but because of the raw materials used to make the medium this would not be considered unusual. Both infusion media were about twice as efficient as TSA for recovering non-heat injured spores. PIT agar

recovered approximately three times as many dry-heat injured spores as did TSA. Table 5 shows that PPA was about equal to TSA and pork and pea broth (PPB) for detecting injured spores. With uninjured spores PPA was slightly more efficient than TSA but about the same as PPB.

When PPA and PPB were compared for recovering injured anaerobic spores of Clostridium sp. SC-1 (Table 6), PPB appeared to be slightly more sensitive, but there was no significant difference with non-injured spores. No differences were found when liquid and solid trypticase soy media were compared. Although these results were typical, there was much variation from test to test, especially with pork and pea media.

These results indicate that the present techniques used for the assessment of microbial contamination on space hardware are practical and valid. Some improvements might be made if different media were employed, but there is no evidence from our studies or in the literature that any medium is so superior to TSA that it should be considered for routine use. PIT is, at most, about 3 to 4 times more efficient than TSA for recovering dry-heat injured anaerobic spores. But considering the expenditure of time and money, and more importantly, the variation that would occur among batches, there would be no advantage in using it routinely for enumeration. If any medium were found to be 10 to 100 times more efficient than TSA, the problem would need to be reconsidered. Also, there seems to be no particular advantage in employing a liquid recovery system for enumeration of spores. Table 6 shows that PPB had an MPN about 3 times as high as the viable count with PPA and that the difference was significant statistically. The MPN listed in the table represented the mean of five MPN values but in practice only one set of multiple tube dilutions would be available. Even though the mean MPN for five series of tubes was higher than the viable counts, in most cases the limits of the 95% confidence interval of the individual MPN's encompassed the mean of the viable count.

Current research designed to improve recovery media might well be directed toward developing supplements which can be added to dehydrated culture media. For the recovery of injured spores it is suggested that the criterion for selecting an assay medium other than TSA should be that the new medium is at least 10 times as efficient as TSA.

5. Under a contract with Westinghouse Electric Corporation a bioclean room was assembled in the support facility at the Phoenix Field Station. Performance tests conducted on the room revealed that several design specifications could not be met. Accordingly, the replacement or modification of certain systems in the room will be made by Westinghouse before the room is accepted and becomes operable.

6. Monitoring of microbial contamination was continued in Hangars AE, AO, and S, the Surveyor sterilization and assembly laboratory, the Surveyor fuel loading room, the Lunar Orbiter camera room, and the Lunar Orbiter fuel loading room. In a cooperative study with the Boeing Company, detachable stainless steel strips were placed on Lunar Orbiter 3 in Seattle and then

removed and assayed at various times after arrival at the Eastern Test Range. Each time that strips were removed and assayed, swab samples also were taken from the spacecraft. Swabbing removed 7.3 times as many microorganisms per unit area from horizontal surfaces as compared to vertical surfaces (Table 7). An average of 157 microorganisms/in.^{2} was recovered from horizontal surfaces by swabbing. An average of 112 microorganisms/in.^{2} of exposed horizontal surface was recovered from the detachable strips.

Contamination levels of the Mariner 67 spacecraft, shroud, and adapter were determined using the swab-rinse technique. The results are presented in Table 8. This study was undertaken in cooperation with the Jet Propulsion Laboratory.

Levels of microbial contamination on the Surveyor 3 spacecraft, shroud and adapter were determined before and after each major spacecraft systems test using the swab-rinse technique (Table 9). The same assay technique was used to determine contamination levels on the Surveyor 4 spacecraft, shroud and adapter (Table 10) and the Lunar Orbiter 3 shroud.

Colonies which developed on TSA during anaerobic incubation (Brewer jars) were subcultured and the numbers which were facultative or strictly anaerobic were determined. Each type of organism isolated was streaked onto two plates of TSA. One was incubated aerobically and the other anaerobically. Of 821 microorganisms isolated from six different areas 783 (95.4%) grew under both aerobic and anaerobic conditions (Table 11).

A number of microorganisms isolated from the surfaces of spacecraft were gram stained and their morphology observed. Results are shown in Table 12.

The contract for the addition to the Sterility Control Laboratory was awarded to the J. C. Abbott Co. for $61,950. Construction was scheduled to start on June 20. The facility will be completed by September 30, 1967.

TABLE 1. EFFECT OF EXTENDED INCUBATION ON NATURALLY-OCCURRING SPORES FROM SOIL EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY AGAR.

Minutes of exposure	Dilution	Mean number[1] of visible colonies per plate after incubation at 32 C							
		No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14	No. of days 21	No. of days 28
22.5	none	49.6	75.9	75.9	Sp.[2]				
	10^{-1}	4.6	8.8	10.9	11.8	13.1	13.1	13.1	13.1

[1] Each value is the mean colony count from nine samples.

[2] Colonies coalesced and could not be counted accurately.

TABLE 2. EFFECT OF EXTENDED INCUBATION ON NATURALLY-OCCURRING SPORES FROM SOIL EXPOSED TO 600 MG/L OF ETHYLENE OXIDE AND RECOVERED IN TRYPTICASE SOY BROTH.

Minutes of exposure	Dilution	No. of tubes	Number of tubes showing visible growth after incubation at 32 C							
			No. of days 1	No. of days 2	No. of days 3	No. of days 4	No. of days 7	No. of days 14	No. of days 21	No. of days 28
22.5	none	19	19							
	10^{-1}	19	19							
	10^{-2}	19	8	16	17	17	19			
	10^{-3}	19	0	5	8	8	9	10	11	12

TABLE 3. TEST MEDIA FOR DRY-HEAT INJURY STUDIES

1. Trypticase soy agar (BBL) dehydrated

2. Pork and pea medium (modified after A. A. Anderson. 1951. A rapid plate method of counting spores of Clostridium botulinum. J. Bacteriol. 62:425-432.)

   Pork infusion.....800 ml

   Pea infusion.....200 ml (fresh frozen peas)

   Peptone (Difco)..... 5 g

   Tryptone (Difco)..... 1.6 g

   K_2HPO_4..... 1.25 g

   Soluble starch (BBL)..... 1.0 g

   Sodium thioglycolate..... 0.5 g

   pH adjusted to 7.2 with NaOH

   For solid medium 15 g of agar was added.

3. Pork infusion thioglycolate medium (modified after E. Staten Wynne and Jackson W. Foster. 1948. Physiological studies on spore germination with special reference to Clostridium botulinum. 1. Development of a quantitative method. J. Bacteriol. 55:61-73).

   Pork infusion.....1,000 ml

   Peptone (Difco)..... 5 g

   Tryptone (Difco)..... 1.6 g

   K_2HPO_4..... 1.25 g

   Soluble starch (BBL)..... 1.0 g

   Sodium thioglycolate..... 0.5 g

   pH adjusted to 7.2 with NaOH.

   For solid medium 15 g of agar was added.

TABLE 4. COMPARATIVE EFFICIENCY OF SEVERAL MEDIA IN RECOVERING SPORES OF CLOSTRIDIUM SP. SC-1.

Treatment	Number of spores producing colonies after incubation at 32 C for 72 hours			
	Trypticase soy agar	Pork and pea agar; batch 1	Pork and pea agar; batch 2	Pork infusion thioglycolate agar
none[1]	970[2]	1,540	1,720	1,730
dry heat[1]	1,940	4,770	5,833	6,070

[1] For both heated and non-heated samples appropriate dilutions were made to yield surviving spore concentrations which would be countable. Consequently, there is no numerical relationship between the treated and non-treated samples.

[2] Each value is the mean colony count from triplicate samples.

TABLE 5. COMPARATIVE EFFICIENCY OF ONE LIUQID AND TWO SOLID MEDIA FOR RECOVERING SPORES OF *CLOSTRIDIUM* SP. SC-1.

Sample	Heated[1]			Non-heated[1]		
	Trypticase soy agar	Pork and pea agar	Pork and pea broth	Trypticase soy agar	Pork and pea agar	Pork and pea broth
	No. of colonies	No. of colonies	Visible growth	No. of colonies	No. of colonies	Visible growth
1	185	359	positive	67	176	positive
2	104	165	positive	35	83	positive
3	44	71	positive	17	43	positive
4	25	50	positive	7	30	positive
5	13	18	positive	5	13	positive
6	4	12	positive	2	6	positive
7	4	3	positive	1	3	positive
8	2	3	positive	0	4	positive
9	1	0	positive	0	1	positive
10	0	1	negative	0	1	negative

[1] For both heated and non-heated samples appropriate dilutions were made to yield surviving spore concentrations which would be countable. Consequently there is no numerical relationship between the treated and non-treated samples.

TABLE 6. COMPARATIVE RECOVERY OF CLOSTRIDIUM SP. SC-1 SPORES USING SOLID AND LIQUID MEDIA.

Medium	Treatment	Plate count no./ml	Multiple tube dilution MPN/ml
Trypticase soy medium	none[1]	510	352
	heat[1]	9.4	3.7
Pork and pea medium	none	1,346	2,344
	heat	840	2,335[2]

[1] For both heated and non-heated samples appropriate dilutions were made to yield surviving spore concentrations which would be countable. Consequently, there is no numerical relationship between the treated and non-treated samples or the two media.

[2] Significant difference; $P < 0.05$ based on student t test is considered significant.

TABLE 7. COMPARATIVE LEVELS OF MICROBIAL CONTAMINATION DETECTED ON THE SURFACE OF LUNAR ORBITER 3 BY STRIPS AND SWABS.

STRIPS[1]					
Area sq.in.	Date	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
40.0	3-10-67	5,150	2,130	340	340
40.0	4-10-67	305	105	40	5
44.0	4-27-67	5,250	110	70	0

[1] All strips were in a horizontal position.

SWABS						
Area sq.in.	Date	Position[2]	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
25.0	3-10-67	H	2,160	700	0	0
32.1		V	415	95	5	5
25.0	4-10-67	H	700	45	0	0
32.1		V	50	50	0	0
25.0	4-27-67	H	190	25	80	15
32.1		V	40	15	5	5

[2] H = horizontal; V = vertical

TABLE 8. LEVELS OF MICROBIAL CONTAMINATION ON THE SURFACE OF MARINER 67-1[1] SPACECRAFT, SHROUD AND ADAPTER AND MARINER 67-2[2] SPACECRAFT, SHROUD AND ADAPTER.

Part sampled	Area sq.in.	Date	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Mariner 67-1 spacecraft	156	4-28-67	370	140	5	0
Mariner 67-1 adapter	80	5-31-67	1,090	550	265	105
Mariner 67-1 shroud	80	5-31-67	135	60	65	20
Mariner 67-2 spacecraft	180	5-2-67	1,045	350	10	0
Mariner 67-2 spacecraft	180	5-10-67	805	130	135	30
Mariner 67-2 spacecraft	224	5-17-67	3,020	485	10	10
Mariner 67-2 spacecraft	180	5-26-67	3,920	1,005	30	10
Mariner 67-2 spacecraft	80	5-28-67	35	20	10	0
Mariner 67-2 adapter	40	5-28-67	2,550	755	5	0
Mariner 67-2 shroud	40	5-28-67	125	70	0	0

[1] Mariner 67-1 is the backup model.

[2] Mariner 67-2 is the flight model.

TABLE 9. EFFECT OF VARIOUS SPACECRAFT SYSTEMS TESTS ON THE SURFACE MICROBIAL CONTAMINATION LEVELS OF SURVEYOR 3.

Part sampled	Date	Area sq.in.	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
ARRIVAL AT THE EASTERN TEST RANGE:						
Spacecraft	2-16-67	80	0	0	0	0
PRIOR TO J-FACT (FLIGHT ACCEPTANCE TEST):						
Spacecraft	2-27-67	80	605	60	0	0
AFTER J-FACT:						
Spacecraft	2-28-67	80	480	175	5	0
PRIOR TO ATLAS-CENTAUR MATCH MATE:						
Spacecraft	3-2-67	80	1,320	245	0	5
Adapter	3-2-67	40	5,330	3,535	10	0
Shroud	3-2-67	40	2,375	90	0	0
AFTER ATLAS-CENTAUR MATCH MATE:						
Spacecraft	3-7-67	80	1,985	435	5	0
Adapter	3-8-67	40	1,205	130	10	0
Shroud	3-8-67	40	2,380	170	25	0
PRIOR TO ALIGNMENT:						
Spacecraft	3-10-67	80	1,140	35	0	5
AFTER ALIGNMENT:						
Spacecraft	3-14-67	80	170	70	5	0
PRIOR TO FUELING:						
Spacecraft	3-21-67	80	395	50	5	20

TABLE 9. EFFECT OF VARIOUS SPACECRAFT SYSTEMS TESTS ON THE SURFACE MICROBIAL CONTAMINATION LEVELS OF SURVEYOR 3 (Continued)

Part sampled	Date	Area sq.in.	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
AFTER FUELING:						
Spacecraft	3-29-67	80	1,160	70	15	5
PRIOR TO RETRO ROCKET AND ALTITUDE MONITORING RADAR MATING:						
Retro rocket	3-31-67	80	125	30	10	0
AMR	4-4-67	80	835	435	75	35
PRIOR TO ADAPTER MATING:						
Spacecraft	4-6-67	80	10,865	1,485	35	25
AFTER ADAPTER MATING:						
Spacecraft	4-7-67	80	9,680	1,110	60	10
PRIOR TO FINAL CLEANING:						
Spacecraft	4-11-67	80	7,945	665	30	30
Adapter	4-11-67	40	1,770	350	45	10
Shroud	4-11-67	40	1,065	110	5	5
AFTER FINAL CLEANING:						
Spacecraft	4-11-67	80	3,400	235	20	0
Adapter	4-11-67	40	165	30	0	0
Shroud	4-11-67	40	195	115	10	5

TABLE 10. MICROBIAL CONTAMINATION DETECTED ON THE SURFACE OF THE SURVEYOR 4 SPACECRAFT, ADAPTER, AND SHROUD AND THE LUNAR ORBITER 3 SHROUD.

Part sampled	Date	Area sq.in.	Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Surveyor 4 spacecraft	4-26-67	80	15	0	0	0
Surveyor 4 spacecraft	5-23-67	80	195	10	10	0
Surveyor 4 spacecraft	5-26-67	80	3,375	30	845	0
Surveyor 4 adapter	5-29-67	40	2,180	735	60	10
Surveyor 4 shroud	5-29-67	32	545	185	0	0
Surveyor 4 spacecraft	6-3-67	80	260	20	10	0
Surveyor 4 adapter	6-3-67	40	1,595	20	265	0
Surveyor 4 shroud	6-3-67	32	1,240	5	615	0
Lunar Orbiter 3 shroud	5-31-67	80	25	10	0	0

TABLE 11. PERCENTAGE OF ANAEROBIC ISOLANTS SHOWING FACULTATIVE CHARACTERISTICS.

Area	Total no. isolants[1]	Facultative	Strictly anaerobic	Percent facultative
Hangar AE	6	5	1	83.3
Hangar AO	41	40	1	97.6
Hangar S	61	59	2	96.6
Area 5/6 fuel loading room	17	3	14	17.6
Area 60-A fuel loading room	394	379	15	96.1
Area 60-2 sterilization & assembly laboratory	302	297	5	95.0
TOTAL	821	783	38	95.4

[1] All microorganisms were detected on stainless steel strips exposed to the various intramural environments and initially grew out in Brewer jars.

TABLE 12. STAINING AND MORPHOLOGICAL CHARACTERISTICS OF MICROORGANISMS ISOLATED FROM THE SURFACES OF SURVEYOR AND LUNAR ORBITER SPACECRAFT.

Source	No. of isolants	Staining and morphology	Percent
Surveyor	239	gram-positive cocci	71.5
		gram-positive rods	23.4
		gram-negative cocci	0.0
		gram-negative rods	2.9
		yeasts, mold, actinomycetes	2.2
Lunar Orbiter	88	gram-positive cocci	44.1
		gram-positive rods	19.1
		gram-negative cocci	0.0
		gram-negative rods	32.4
		yeasts, mold, actinomycetes	4.4